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THE LABORATORY PRODUCTION OF GONYAULAX CATENELLA POISON

CHEMICAL CORPS BIOLOGICAL LABORATORIES
Camp Detrick, Frederick, Maryland

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THE LABORATORY PRODUCTION OF GONYAULAX CATENELLA POISON

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SPECIAL REPORT NO. 174

THE LABORATORY PRODUCTION OF GONYAULAX CATANELLA POTSON

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SPECIAL REPORT NO. 17

THE LABORATORY PRODUCTION OF GONYAULAX

CATANELLA POISON

SUMMARY

Investigations on the laboratory production of Gonyaulax catanella poison were carried out at Camp Detrick from April 1951 to March 1952. Methods were developed for the estimation of growth by direct microscopic count or by the analytical determination of cellular carbohydrate. Poison was extracted from G catanella cells and assayed as shellfish poison. Use of a large inoculum, illumination with fluorescent light, and continuous incubation at 17 C for 7 to 14 days gave maximal yields of 50,000 to 80,000 cells per ml in sea water and in artificial sea water media. Improved growth was obtained by increasing the nitrate and iron concentration in sea water medium. The addition of culture filtrates, extracts of contaminant bacteria or organic nutrients to sea water media did not significantly alter growth of G catanella. Contact with rubber products or galvanized sheet metal completely inhibited growth. Stainless steel or polyethylene caused slight inhibition which was overcome with ethylenediaminetetraacetate (EDTA). Saran plastic or aluminum did not inhibit growth. Artificial sea water media prepared with a mixture of inorganic salts (Lyman and Fleming), or with commercial sea salt dissolved in tap water, supported good growth of sea water inocula but failed to support G catanella growth after repeated subculture. Growth in the sea salt medium was improved by the addition of 0.01 per cent EDTA and 5 to 15 per cent of sea water. The contaminants in the stock G catanella culture were not eliminated by treatment with antibiotics, washing processes, or ultraviolet irradiation. The addition of polymyxin B, penicillin, streptothricin or streptomycin to culture media improved the growth of G catanella. The best poison yield, 1.3 mouse units (MU) per ml, in production trials was obtained in 3.5 liters of sea water culture containing 17 µg of polymyxin B per ml after 7 days of incubation in a rotating fermentor with continuous fluorescent illumination. With an average yield of 1 MU per ml per week, the estimated poison production with the available facilities was 70,000 MU per week.

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I. INTRODUCTION

The nutrition of the plankton dinoflagellate, Gonyaulax catenella was studied at Camp Detrick from April 1951 to March 1952. A stock culture, originally isolated from "red water" collected in Monterey Bay in May 1949, was obtained from the investigators at the University of California studying shellfish poison under Contract W-[illegible]-ORL-OM-251. The culture was free of other plankton but contained microbial contaminants. Using this culture the California investigators had developed several media containing aged or autoclaved Pacific water supplemented with marine mud (soil) extract and inorganic sources of nitrate, phosphate, iron and silicate. Six volumes of medium were inoculated with 1 volume of culture and the cultures (87 to 7,525 ml volumes) were incubated in pyrex bottles in a 17 C water bath for an optimum growth period of 1 week. The cultures were illuminated with fluorescent or incandescent lights so placed that the light was filtered through 1/2 to 1 inch of water. Maximum yields of 2,000 to 14,000 cells per ml (with the exception of one yield of 40,600 cells per ml) were obtained. The cells were centrifuged from the culture medium and extracted for poison with boiling 0.1 N HCl and the extracts were assayed by the standard mouse assay method for mussel poison used in Project 4-61-14-001. The maximum poison yield obtained by the California workers was 1.2 mouse units per ml for a culture containing 40,600 cells per ml. The expected production with large scale equipment (20 liter bottles) devised by these workers was 5,000 to 10,000 mouse units per week.

The investigations described in this report were undertaken with the objective of providing a reliable source of G catenella poison in order that chemical characterization might proceed without interruption caused by failure of the natural sources. Attempts were made to increase poison yields by systematic studies on nutrition and incubation conditions affecting G catenella. Unfortunately, the results of some of the experiments are not as complete as desired. Progress was retarded by the slow growth and fragility of G catenella as well as by the presence of contaminants in the stock culture, which interfered in the evaluation of nutritional effects. The abrupt termination of the project interrupted plans for additional studies and left some experiments partially completed. This work was included for its possible use to future investigations of G catenella.

2. THE EVALUATION OF GONYAULAX CATANELLA CULTURES

a. METHODS FOR THE DETERMINATION OF GROWTH

(1) Direct Microscopic Cell Counts

Gonyaulax catanella is a unicellular, pigmented dinoflagellate occurring singly and in short chains of 2 to 4 cells in artificial culture. Each cell is approximately 30 to 40 microns in diameter in artificial culture. Microscopic cell counts were made by the California workers on culture samples diluted 1 to 100 with 2.5 per cent formalin. In the investigations at Camp Detrick approximately 0.035 ml of a well-mixed undiluted culture was transferred to a Howard mold counting chamber; the chamber was heated for 1 minute at 70 C to inactivate the motile cells and placed on the stage of a binocular microscope giving 100X magnification. The intact cells in 30 or more fields were counted at approximately equally spaced locations by moving the chamber with a mechanical stage. The average count per field multiplied by the reciprocal of the field volume in ml (5300 for Spencer binocular microscope with 10X objective and 10X eyepiece lenses) gave the number of cells per ml. Duplicate or triplicate samples of each culture were counted. The averaged count of 2 or more samples was recorded as the cell yield. Replicate counts usually did not vary by more than 20 per cent. The small sample volume and the tendency for uneven cell distribution on the counting chamber limited the accuracy of this method.

(2) Visual Estimation of Growth

It was possible to estimate G catanella growth visually in cultures containing more than 1000 cells per ml. This method was convenient for following growth in test tube cultures throughout the incubation period without disturbing the culture by frequent removal of samples. Growth was expressed by the numerals 0 through 5 which had the following approximate relation to cell counts:

Visual	Cells per ml
0	0 to 1,000
1	1,000 to 5,000
2	5,000 to 15,000
3	15,000 to 25,000
4	25,000 to 40,000
5	40,000 or more

Visual estimation of the suspended growth in unmixed cultures gave an approximation of the number of living cells, an indication of culture vigor

because dead cells settled out. Intact dead cells were not distinguished from living cells by the microscopic count.

(3) Determination of Cellular Carbohydrate

The anthrone method[1], a quantitative colorimetric determination for carbohydrates, was studied as a possible method for determining cell numbers of G catanella. The cells were separated from the medium by centrifugation, treated with the anthrone reagent, and the resulting blue color measured photometrically. Samples of 1, 5, 10, 20 and 40 ml volumes of a well mixed 16-day-old culture of G catanella grown in 500 ml of sea water medium were transferred to 40 ml conical centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The carbohydrate content of the supernatant liquid was found to be negligible and it was decanted and drained by placing the inverted tubes on filter paper. The cells in the tube were resuspended in 3 ml of distilled water and the tubes placed in an ice-water bath. Seven ml of the anthrone reagent (0.2 per cent anthrone in 95 per cent H_2SO_4) was added to each tube slowly with shaking to avoid overheating during the mixing. The tubes were heated in a boiling bath for 10 minutes and cooled. The optical density (OD) of the clear blue color was measured in a Coleman spectrophotometer at 620 mμ. The following results were obtained:

Culture volume, ml	Total cells, thousands	OD	OD/1,000 cells
1	3	0.0555	0.0185
5	15	0.278	0.0185
10	30	0.589	0.0196
20	60	1.204	0.0201

A linear relationship between optical density and the number of cells analyzed is shown by the agreement among the OD values per 1000 cells. These values also establish the sensitivity of the method to be approximately 100 cells, which is greater than the direct cell count method. Disadvantages of the anthrone method were that it did not distinguish between intact cells and cellular debris as was possible by the direct count method, and that the OD value per 1000 cells varied with culture age.

1. Neish, A. C., 1950, Analytical Methods for Bacterial Fermentations, National Research Council of Canada, Report No. 46-8-3, Revised, p.30.

(4) Determination of Cellular Pigment

Spectrophotometric examination of the pigments extracted from C catenella cells with methanol showed major absorption maxima at 440 and 675 mμ. To determine whether a correlation existed between the number of cells extracted and the absorption values of the pigment at 440 mμ, different volumes of a culture containing 39,000 cells per ml were filtered on a 1.5 cm sintered glass filter. The cells on the filter were washed once with 1 ml of water and extracted at 25 C with five 1 ml volumes of 90 per cent methanol. The following optical density values were obtained with duplicate extracts:

Culture volume, ml	Cells extracted thousands	Optical density	
1	39	0.015	0.01
3	117	0.04	0.055
4	156	0.07	
5	195	0.143	0.095
7	273	0.180	

The method was unsatisfactory for the quantitative determination of growth because the optical density values of duplicate samples varied and because the optical density did not increase in proportion to the number of cells extracted. These errors were probably caused by inequalities in pigment extraction. With improved accuracy this method of determining pigmented (predominately viable) cells might provide better estimation than the anthrone method which was subject to errors due to cellular debris and contaminant growth.

Additional absorption maxima were observed in pigment extracts of the cells from 3.5 liters of culture. The cells were extracted in a centrifuge tube with 6 successive 2 ml volumes of absolute methanol. The pigments were extracted from the cells at different rates, i.e., orange and red (xanthophyllic) pigments predominated in extracts 1 and 2, green (chlorophyllic) pigments in 3 and 4, and yellow pigments in 5 and 6. The absorption maxima of the extracts were determined with a Beckman model DU spectrophotometer. They are listed in order of decreasing intensity for each extract, as follows:

Extract	Color	Absorption maxima, (mμ)
1	orange-red	328
2	red-green	328, 270, 420, 675
3	green	675, 410, 330, 540, 510
4	green-yellow	675, 410, 620, 540, 510
5	yellow-green	410, 675
6	yellow (slight green)	410, 675

The strong absorption of blue light and red light may be of significance in selecting optimum illumination conditions for the artificial cultivation of G catanella.

(5) Determination of Culture Turbidity

The turbidimetric method was a rapid means of evaluating growth in cultures where extraneous turbidity caused by contaminant growth or precipitates in the medium was absent. A linear relationship was shown to exist between the cell count and the turbidity values of culture samples. A Coleman photonephelometer was used to measure the turbidity of culture samples in 18 mm test tubes. The instrument was adjusted to 0 turbidity with the appropriate uninoculated medium. The cells in the culture sample were evenly suspended before reading by tapping the tube lightly, and the first steady galvanometer reading was recorded as the turbidity value. The steady value decreased after about 30 seconds as the cells either settled or swam to the surface of the liquid. The disadvantages of this method were its failure to give reproducible turbidities and the fact that dead cells, cell fragments, and contaminant growth were included in the turbidity values. A more accurate estimation of viable growth was possible by visual examination of the culture, and the visual method was used in preference to the turbidimetric method.

b. THE DETERMINATION OF POISON YIELDS

The growth (cell yields) and toxicity of G catanella cultures often were not parallel; therefore, it was necessary to determine poison yields for accurate evaluation of media to be used for poison production. The poison was extracted from the G catanella cells and determined by the standard mouse assay method for shellfish poison. Results were expressed as mouse units (MU) per ml, a mouse unit being the amount of poison which kills a 20 gram mouse 15 minutes after intraperitoneal injection. The poison yield did not include poison released from cells into the culture medium during

incubation. The amount of poison lost in this manner was not established, since a method for the recovery of poison from sea water medium was not available, and the poison could not be assayed accurately in the medium.

The selection of a poison extraction method was based on the results of tests with different extraction solvents and different methods of collecting and treating the cells for extraction. The extraction solvents investigated were: 0.1 and 0.01 N HCl, 0.01 N acetic acid, 0.01 N HCl containing 15 per cent ethanol, 10 per cent trichloroacetic acid (TCA), and water. The cells from 25 to 100 ml culture samples were extracted at 80 to 95 C with successive portions of the solvent. Several tests were made with different culture samples at different times in this investigation. A 0.01 N HCl extract was prepared in each solvent test to give a baseline for comparing the efficiency of solvents in different tests. The following data show that the best extraction solvents were 0.01 N HCl and 10 per cent TCA:

Solvent	Test solvent MU per ml	0.01 N HCl control MU per ml
0.1 N HCl	0.22	0.22
	0.10	0.14
15% ethanol in 0.01 N HCl	0.07	0.10
	0.15	0.21
0.01 N acetic acid	0.07	0.09
	0.03	0.05
10% TCA	0.52	0.45
Water	0.02	0.05

The TCA extract was obtained by a single extraction with 1 ml of 10 per cent TCA followed by extraction with 2 ml of water. The TCA was removed from the extracts by treating the mixture with 1 gm of anion exchange resin (IR-4B) to give a neutral filtrate for assay. The poison extracts obtained with the other solvents did not require treatment other than dilution before assay. Based on poison yields and convenience of use, 0.01 N HCl was selected as the poison extraction solvent for this Project.

A convenient and rapid procedure was desired for poison extraction from the numerous experimental cultures. A suitable procedure consisted of collecting the cells from 100 ml or less of culture on a layer of celite on a Hirsch funnel (size 0000) by vacuum filtration. The filter containing the cells was placed in an oven at 80 to 100 C and the cells extracted with one 1 ml and four 0.5 ml volumes of 0.01 N HCl added at 2 minute intervals. The extracts were collected and brought to the desired volume in calibrated 12 ml centrifuge tubes. An alternate method consisted of

collecting the cells in tapered 40 ml centrifuge tubes by centrifuging for 5 minutes at 2000 rpm. The supernatant liquid was poured off; 0.01 N HCl was added; the sediment was resuspended; and the tube was placed in a boiling water bath for 10 minutes. The tube was centrifuged for 5 minutes at 3000 to 4000 rpm and the extract was removed with a pipette. The acid extraction was repeated several times. The two methods gave similar results with culture samples containing less than 0.2 MU per ml; however, when the toxicity exceeded this value, higher poison yields were obtained by extraction in centrifuge tubes as shown by the following data:

Sample	Filter method MU per ml	Centrifuge tube method MU per ml
1	0.05	0.07
2	0.08	0.06
3	0.14	0.14
4	0.16	0.39
5	0.28	0.45

Improved poison yields were obtained by reducing the amount of celite used in the filter method. Sample number 4 yielded 0.16 MU per ml when filtered and extracted on a layer of celite 1/4 inch in depth. This yield was increased to 0.21 MU per ml with 1/8 inch of celite, and to 0.33 MU per ml when no celite was used. However, the filtration of culture samples without celite was frequently slow, and the centrifugation method was preferred for routine test assays. A modification of this method was used to extract the poison from cultures of 4 to 11 liter volumes. The cells were collected in a Buchner funnel containing a thin layer of celite on a coarse filter paper (Eaton-Dikeman No. 615). The cell and celite mixture was transferred to a centrifuge tube and extracted by the above procedure.

A single attempt was made to collect and extract cells on a celite column as reported by the California investigators (Contract Number W-18-064-CM-251, 15 Apr 51). A 1000 ml sample of culture was passed through a column of celite 2 inches in diameter and 4 inches deep. The column was placed in a 57 C oven and extracted with three, 25 ml volumes of boiling 0.01 N HCl. The combined extracts gave a yield of 0.043 MU per ml as compared to 0.069 MU per ml by 3 extractions of a sample of the culture in a centrifuge tube.

Microscopic examination showed that most of the C cavanella cells retained intact cell walls after extraction with hot 0.01 N HCl or 0.1 N HCl. Attempts were made to break up the cells in order to improve poison

extraction. Very few intact cells remained after oscillation for 10 minutes with 0.1 or 0.2 mm diameter glass spheres and 0.01 N HCl in the cup of a Mickel disintegrator. The mixture of acid, disintegrated cells, and glass spheres was heated for 10 minutes and filtered. A poison extract of the same culture by the filter method served as the control. The results did not show increased poison yields by the disintegration process:

Sample	Filter MU per ml	Disintegration MU per ml
1	0.14	0.18
2	0.20	0.16
3	0.10	0.10
4	0.08	0.06

The effect of the presence of a plasmolyzing agent on poison extraction was studied by including 1 per cent of ethyl acetate with the first portion of extraction solvent. The results with 3 solvents showed no improvement in poison yields by ethyl acetate treatment:

Solvent	Control MU per ml	1% ethyl acetate MU per ml
0.01 N HCl	0.090 0.045	0.080 0.050
0.01 N acetic acid	0.070 0.029	0.050 0.030
Water	0.020	0.020 to 0.026

A lack of correlation between yields of cells and poison was often observed. Deterioration of poison within the cells, or diffusion of poison from the cells into the medium during the growth period would result in reduced toxicity. A loss of cellular poison was demonstrated for cells suspended in sea water and stored in the dark at 5 C or 17 C to minimize growth and poison synthesis. The cells in a 250 ml sample of culture (containing 51,000 cells per ml) were collected by gravity filtration on coarse filter paper and resuspended in 250 ml of sterile sea water. The resulting suspension containing 46,000 cells per ml was mixed and divided into five 50 ml samples. Duplicate samples were stored in the dark at 5 C and 17 C. The

remaining 50 ml sample was extracted for the initial (0 days) poison yield. After 3 and 7 days, samples stored at each temperature were removed and extracted. Poison yields and cell counts decreased during storage at both temperatures. The increased numbers of cells per MU indicated that the poison yield decreased more rapidly than the cell yield:

Storage days	17 C			5 C		
	Cells/ml (thousands)	MU/ml	Cells/MU (thousands)	Cells/ml (thousands)	MU/ml	Cells/MU (thousands)
0	46	0.17	270	46	0.17	270
3	38	0.10	380	40	0.11	365
7	33	0.08	412	31	0.10	310

The cells stored at 17 C had lost 25 per cent of their initial poison content after 3 days and more than 33 per cent after 7 days of storage. Similar losses in growing cultures would materially reduce poison yields. It would be desirable to minimise such loss by a continuous harvesting of the cells or by devising methods for the recovery of the poison lost to the culture medium.

3. CONDITIONS FOR THE ARTIFICIAL CULTIVATION OF GONYAULAX CATANELLA

a. INOCULUM

Cultures of G catanella incubated for 1 to 2 weeks in 1 liter of sea water medium in Erlenmeyer or low form culture flasks were used as a standard inoculum (sea water inoculum) for experimental and production media. In the early part of the study, whole cultures containing both living and dead cells were used as inocula. Later it was found that an inoculum consisting only of actively motile cells produced a shorter growth cycle. The motile cells at the surface of the culture were concentrated by carefully decanting the top layer of the culture into a sterile 500 or 1000 ml cylinder or aspirator bottle. The most active cells again rapidly swam to the surface of the liquid. When a small volume of highly concentrated motile cells was desired, the lower layer of the pooled inoculum in the cylinder or aspirator bottle was slowly syphoned off. Total cell counts were made on all cell suspensions used as inocula. When a series of media was inoculated the pooled inoculum was mixed frequently to insure the transfer of equal numbers of cells to each experimental medium. All media was brought to 17 C before inoculating.

The effect of inoculum size on growth in different volumes of sea water medium was studied. A 16 or 20 per cent inoculum was superior to a 10 per cent inoculum. Increasing the inoculum from 10 to 20 per cent approximately doubled the cell yield in 50 ml volumes of medium. Increasing the

inoculum from 16 to 20 per cent gave increased cell yields after 2[illegible] days of incubation in 1,000 ml volumes of medium. The summarized data were:

Experiment	Volume ml	Inoculum per cent	Replicate cultures	Cells per ml (thousands) Days of incubation 3	5	7	9	12	14	20
1	50	10	2	6.6	11			17		
	50	20	2	13				45		
2	1000	16	3	4.6	8.5		11		19	25
	1000	20	3	5.2	12		15		17	38

A 16 per cent inoculum (1 volume of inoculum into 6 volumes of medium) was selected for routine use.

b. CULTURE VESSELS

All cultures were incubated at 17 C either in a constant temperature laboratory or a water bath. A variety of culture vessels was used with different volumes of media: 5 or 10 ml volumes of culture were incubated in 18 mm test tubes, 50 or 100 ml in 250 ml Erlenmeyer flasks, 100 ml in 500 ml Erlenmeyer flasks or in 2.5 liter low form culture flasks, 200 or 600 ml in 1 liter beakers, and 3.5 to 11.5 liters in 20 liter bottles. All glassware was pyrex. The cultures in test tubes or flasks were usually closed with cotton plugs, although aluminum or glass caps were sometimes used. Watch glasses or sheets of cellophane were used on beakers.

c. ILLUMINATION

(1) Source and Intensity

C catanella requires illumination for growth. Fluorescent light provided a convenient source of illumination and was used exclusively. Cultures in test tubes gave similar growth when illuminated by any of the following methods: (1) in air at 17 C, 1 inch above three 40-watt tubes; (2) in an 8 inch round water bath surrounded by a 32-watt Circline tube; or (3) in a 20 by 38 inch water bath 0.5 to 1 inch above a submerged 20 watt tube. Method (1) gave an illumination intensity of approximately 1000 foot-candles and methods (2) and (3) gave approximately 300 foot-candles. Good growth was obtained in larger volumes of culture illuminated by fluorescent lights placed above, beneath or beside the cultures. Cultures in flasks and beakers were usually incubated on shelves covered with

aluminum foil 1 foot below three 40-watt tubes (light intensity of 1000 foot-candles). Cultures in 10.5 and 20 liter bottles were incubated in either an upright position between 2 sets of three 40-watt tubes or in a horizontal position (1) between 2 sets of three 40-watt tubes or (2) with a set of two 15-watt tubes at each side of the bottle and one 15-watt tube below the bottle. Yields of 42,000 to 57,000 cells per ml were obtained in sea water medium cultures in test tubes and flasks incubated under a variety of illumination conditions, indicating that illumination was not a limiting factor in the artificial cultivation of G catanella.

(2) Continuous and Intermittent Illumination

Growth was compared in test tube cultures in sea water media exposed to continuous illumination or to intermittent illumination (16 hours of illumination followed by 8 hours of darkness). Cultures grown with continuous illumination gave larger 11-day yields than the corresponding cultures receiving intermittent illumination.

Medium	Exp. No.	Cells per ml/(thousands) Continuous	Intermittent
Sea water	1	79	31
	2	53	25
	3	54	38
Sea water without soil extract	4	35	18
	5	33	26

The rate of contaminant growth was not reduced by intermittent illumination; therefore, continuous illumination was advantageous for the artificial cultivation of G catanella.

(3) Illumination Through a Water Filter

An indication that artificial light contains wave lengths detrimental to G catanella led the California workers to use a water filter between the culture and the light source. This requirement was not confirmed by experiments in the present work. Test tubes containing 5 ml of culture were incubated 1 inch above a 15-watt fluorescent light. Cultures illuminated by direct fluorescent light gave an average yield of 43,000 cells per ml compared to 42,000 cells per ml in cultures illuminated through 1 inch of water. Similar results were obtained with 3 liter volumes of culture incubated in jars between fluorescent lights. One jar of culture was placed in a jar 2 inches larger in diameter and the annular space was filled with water to provide the filter. This culture gave 6 and 11 day cell yields of 5,000 and 13,000 cells per ml, and a 6 day poison yield of 0.04 MU per

ml. The corresponding yields in the control culture were 7,000 and 16,000 cells per ml, and 0.05 MU per ml.

(4) Penetration of Light into Cultures

A study of G catanella growth in beakers containing different depths of medium was made by preparing sea water medium in 2 duplicate sets of 1 liter beakers. One set contained 200 ml each of medium (culture depth of 2.5 cm), the other set contained 600 ml each of medium (culture depth of 7 cm). One of the duplicate cultures of each set was covered with a watch glass, and the other with a sheet of cellophane. The cultures were incubated under three 40-watt fluorescent lights. Although the cell counts of all the cultures were similar after 9 days of incubation, better poison yields were obtained in the shallow cultures. The poison yields in the cultures illuminated through glass were twice those of the corresponding cultures illuminated through cellophane, suggesting that detrimental wave lengths of fluorescent light penetrated the cellophane.

Culture depth (cm)	Cells per ml (thousands)		MU per ml	
	Glass	Cellophane	Glass	Cellophane
7	18	19	0.22	0.11
2.5	19	25	0.57	0.25

Additional evidence was obtained for the cellophane effect by subculturing samples of the shallow cultures into duplicate 500 ml volumes of sea water medium in 2 liter beakers. A second subculture was made in a similar manner from this subculture after 7 days of incubation. The cell and poison yields after incubating the first subculture for 7 days and the second subculture for 9 days were:

Cover	Cells per ml (thousands)		MU per ml	
	Subculture 1	2	Subculture 1	2
Glass	9	30	0.19	0.30
Cellophane	16	26	0.18	0.15

The second subculture again yielded one half as much poison when illuminated through cellophane, indicating that short wave lengths of light which penetrated cellophane but not glass reduced poison synthesis or poison stability.

(5) Wave Length

The effectiveness of light of different wave lengths was compared by incubating duplicate 5 ml volumes of culture in tubes 0.5 inches above different colored fluorescent lights. The results of an experiment performed with 2 different media under identical conditions showed equal growth with white, red or green light. Blue light supported better growth in sea water medium and less growth in the artificial medium:

Color	Intensity (foot-candles)	Cells per ml (thousands) sea water medium	artificial medium
white (control)	1000	19	21
red	50	17	21
green	575	17	19
blue	500	32	12

The effect of illuminating cultures with mixed white, red, and blue fluorescent light was studied. Three 1,200 ml cultures were incubated between two sets of 3 white fluorescent tubes and 3 similar cultures were incubated between 2 sets of lights, each containing a white, a red, and a blue fluorescent tube. After 10 days of incubation the following growth and poison yields were obtained:

Illumination	Cells per ml (thousands)	MU per ml (pooled cultures)
white	13, 12, 11	0.13
mixed	10, 10, 11	0.26

(6) Growth with Natural Illumination

Studies on the utilization of sunlight as a source of illumination for G catanella were delayed by the lack of equipment to maintain 17 C growth temperature for greenhouse or outdoor cultivation. For example, the death of G catanella cultures, which were incubated in a greenhouse in January 1952, was believed to be attributable to fluctuating temperatures which ranged from 27 to 30 C at mid-day to 13 to 18 C at night. However, the possibility of incubation with indirect sunlight was investigated after cultures of G catanella remained viable for several weeks when placed at room temperature in a north window of the laboratory. Duplicate 2.5 liter low form culture flasks containing 700 ml of sea water medium were incubated at a north window which received approximately 10 hours of indirect sunlight per day. Control cultures were incubated at 17 C with continuous fluorescent illumination (1000 foot-candles). A continuous record of the air temperature around the flasks incubated at room temperature showed

daily fluctuations ranging from 15 to 18 C in the early morning to 24 to 27 C in the late afternoon. The average cell counts per ml of the cultures incubated at room temperature decreased from 3,000 cells per ml after 4 days of incubation to 2,600 cells per ml after 7 days and 1,900 cells per ml after 11 days of incubation. The corresponding cell counts on the control cultures were 6,300, 5,500 and 5,800 cells per ml respectively. The decreasing cell counts were probably caused in part by contaminant growth which was favored by the higher incubation temperature. Improved growth may be possible in media containing antibiotics (polymyxin B, circulin sulfate or streptothricin) to inhibit some of the contaminants, or by artificial illumination of the culture during the dark period of the day to favor growth of G catanella.

d. TEMPERATURE

Growth periods in excess of 2 weeks were often required to obtain maximum cell yields of G catanella at the standard 17 C incubation temperature. Elevating the incubation temperature did not shorten the lag period of growth. Two constant temperature water baths, each illuminated with a 32-watt circline fluorescent tube, were used to incubate duplicate test tube cultures. Separate experiments were conducted to compare growth at 17 C with growth at 20 and 22 C. After 17 days incubation the following results were obtained:

Temperature (C)	Cells per ml (thousands) Sea water medium	Artificial medium
17	69	45
22	16	11
17	17	15
20	13	1

The lower cell yields and the tendency for contaminating bacteria to grow more rapidly at 22 C indicated that a 5 C elevation of temperature was undesirable. Growth in sea water medium at 20 C was equal to that at 17 C, but G catanella did not tolerate a 20 C incubation temperature in the artificial medium.

e. GROWTH IN AERATED CULTURES

G catanella cells showed a tendency to swim to the surface of cultures even though the density of the cells was greater than that of the medium. This behavior in cultures incubated above fluorescent lights (contrary to

the expected phototropic response) suggested that the aeration in the underlying medium was inadequate. Attempts were made to aerate cultures by using a glass sparger or a magnetic stirrer. A static culture was incubated as a control in each experiment. The results with 1.2 and 1.65 liters of culture in 2 liter beakers (experiments 1 and 2) and with 5.8 liters of medium in 10 liter bottles or in 7 liter jars (experiments 3 and 4) were:

Aeration	Cells per ml (thousands)						
	Exp. 1		Exp. 2		Exp. 3		Exp. 4
	7 days	15 days	7 days	10 days	4 days	10 days	9 days
none	11	15	18	27	4	10	10
sparger	13	24	17	19	8	7	6
stirrer	13	21	18	29	7	9	13

Only slight differences in growth were observed, and neither aeration method consistently improved yields. Using a sparger to aerate 11.5 liter volumes of culture in 20 liter bottles likewise failed to improve growth significantly. Aeration by means of a rotating fermenter was more successful and is discussed later in this report.

4. MEDIA FOR THE ARTIFICIAL CULTIVATION OF [redacted]

a. SEA WATER MEDIA

(1) Standard Sea Water Medium

A sea water medium developed by the investigators at the University of California served as the standard sea water medium in these investigations. The composition of this medium was:

autoclaved sea water	97.6 per cent
soil extract	2.0 per cent
KNO_3	100 mg per liter
K_2HPO_4	10 mg per liter
$FeCl_3$	1 mg per liter
Na_2SiO_3	0.005 mg per liter

The sea water was collected, autoclaved, and stored at room temperature in pyrex bottles. Soil extract was prepared by steaming a suspension of marine soil in distilled water (0.5 gm per ml) for 1 hour and filtering

the supernatant liquid through a 1/4 inch layer of celite on a Buchner funnel. The extract was autoclaved and stored at 5 C. The complete medium was autoclaved and cooled to 17 C before inoculation. Sea water medium was used to carry the stock culture and to supply standard inoculum cultures for the experimental studies. Modifications of the sea water medium were tested and are discussed in the following sections of this report.

(2) Source of Sea Water

Satisfactory growth of G catanella was obtained in sea water medium prepared with Atlantic or Pacific sea water or with Atlantic sea water collected at different depths anddistances from the shore. Through the cooperation of the Navy, 200 gal. of sea water was collected in July 1951 from the Atlantic Ocean off the coast of Virginia Beach, Virginia, at different depths from the open sea and near the shore. The following cell yields in 5 ml volumes of sea water medium prepared with samples of this collection were obtained after 14 days of incubation:

Sea Water	Cells per ml (thousands)
9 miles out, surface water	32
9 miles out, 6 feet below the surface	33
9 miles out, 12 feet below the surface	27
1 mile out, 6 feet below the surface	38
In bay, ebb tide, 3 feet below the surface	42

The results show satisfactory growth with each of the sea water samples.

(3) The Effect of Soil Extract

The effect of omitting the marine soil extract from sea water medium was investigated as a possible means of expediting the large scale production of G catanella poison. A preliminary experiment showed equal growth of G catanella, by visual estimation, in the first passage in sea water medium and in sea water medium without soil extract. The inoculum was grown in sea water medium. In a second experiment, growth was determined by direct cell counts. After 2 passages in sea water medium without soil extract neither the normal cell yield of 20,000 to 40,000 cells per ml after 7 to 14 days of incubation nor the normal poison yield of 0.2 to 0.3 MU per ml was diminished. However, repeated passage through the medium without soil extract resulted in the reduction of cell yields to 10,000 to 20,000 cells per ml and in the reduction of poison yield to 0.1 to 0.2 MU per ml. The number of G catanella cells linked in chains was reduced from a maximum of 4 cells per chain to a maximum of 2 cells per chain with a preponderance of single cells. Extracts of soils from various sources were tested for stimulative effects on these soil extract-depleted cells.

Improved growth was obtained with the standard volume of extracts of garden and river soils as well as with marine soils, although the solids content of the extracts varied. River sediment and garden soil were satisfactory substitutes for marine mud as a source of soil extract as shown by the following data:

Source	Per cent solids in medium	Cells per ml (thousands)
No soil	0	38
Pacific mud,[1] tideland area	0.0182	63
Pacific mud, mouth of fresh water stream	0.032	60
Pacific mud, sewage contamination	0.0186	41
River sediment[2]	0.0014	73
Garden soil[3]	0.0014	53

1) The Pacific muds were collected by R. E. Mills of Project W-19-064-CM251.

2) The river sediment was obtained from the Monocacy River at the inlet to the Camp Detrick filtration plant with the assistance of personnel of the Facilities Division.

3) Local garden soil rich in organic matter.

(4) Utilization of Nitrate and Phosphate

Quantitative determinations of inorganic phosphate and nitrate in freshly inoculated sea water medium and in a culture filtrate showed the removal of these nutrients from the medium during growth. A direct correlation between the increase in cells per ml and the decrease in nutrients was found when quantitative determinations of the soluble inorganic phosphorus, total phosphorus and nitrate nitrogen in cultures were performed at intervals during a 2-week growth period. Duplicate 60 ml volumes of sea water medium without soil extract in 250 ml Erlenmeyer flasks were inoculated with 10 ml each of sea water inoculum. A 10 ml sample was removed from each flask and filtered through sintered glass to give samples for the initial values. Additional 10 ml samples were removed after 4, 8 and 14 days and treated as the initial samples. The following averaged results were obtained:

Incubation (days)	Cells per ml (thousands)	Total P (µg per ml)	Inorganic P (µg per ml)	Nitrate N (µg per ml)
0	4.6	2.4	2	15.7
4	12	1.0	1	10.7
8	12	0.9	0.9	8.5
14	25	0.5	0.5	5.3

The correlation between the cell yields and the amount of inorganic nutrient removed from the medium indicated that limiting concentrations of phosphate and nitrate might be reached with continuing growth; therefore, the effect of increasing the phosphate and nitrate concentrations in the medium was studied.

Supplementing the phosphate concentration of sea water medium without soil extract during growth did not increase cell yields of G catanella. After 8 days of incubation, 8,400 cells per ml were present and approximately 50 per cent of the initial inorganic phosphate remained in the medium. At this time the phosphate concentration of half of the cultures was restored to the initial level. After 15 and 19 days of incubation 8,900 to 10,600 cells per ml were present in the non-supplemented cultures and 9,700 to 10,400 cells per ml in the supplemented cultures. There was no further decrease in the total inorganic phosphate concentration in any of the cultures after 15 or 19 days. The inoculum for this experiment was grown in sea water medium without soil extract and the low yields in all cultures may have been due to the effects of soil extract depletion.

The cell yields of G catanella in sea water media containing different amounts of nitrate increased with increased nitrate concentrations up to 0.015 per cent KNO_3. Further increases in nitrate concentration had little effect on growth. The average yields in triplicate flasks containing 100 ml volumes of medium containing 0 to 0.025 per cent KNO_3 were:

Per cent KNO_3	Cells per ml (thousands) Experiment 1		Experiment 2	
	7 days	14 days	7 days	14 days
0	10	6		
0.0025	17	18		
0.005	22	37		
0.01 (control)	24	57	9	39
0.012			14	36
0.015	22	63	7	45
0.02	13	63		
0.025	13	51		

The small increase in the 14 day yields obtained by increasing the KNO_3 concentration from 0.01 to 0.015 per cent was consistent in additional experiments and this modified medium was used in some of the following investigations.

(5) The Effect of Varying the Iron Concentration

The concentration of $FeCl_3$ in the sea water medium was 1 µg per ml. A preliminary experiment testing smaller concentrations of $FeCl_3$ showed

improved 14 day cell and poison yields in a medium containing 0.25 µg $FeCl_3$ per ml and a soil extract prepared from local garden soil. The experiment was repeated using a washed cell inoculum to reduce the carry-over of $FeCl_3$ which may have influenced growth in the low iron media. The effect of adding 0.01 per cent of the metal binding agent ethylene-diaminetetraacetate (EDTA) was also studied. Duplicate 100 ml volumes of each medium in 500 ml Erlenmeyer flasks were inoculated with a sea water suspension of cells which had been collected on coarse filter paper and washed with sterile sea water. The rate of growth was reduced with the washed inoculum, especially in tubes containing the lower amounts of $FeCl_3$. After 14 days of incubation the highest cell yield was found in the control medium, and the yield decreased as the amount of iron decreased. The addition of EDTA improved growth in all of the media; the improvement being more pronounced after 21 days of incubation:

$FeCl_3$ µg/ml	Cells per ml (thousands)			
	14 days		21 days	
	0% EDTA	0.01% EDTA	0% EDTA	0.01% EDTA
0	3.5	12		53
0.001	3.8	7.4		
0.01	4.8	7.2		
0.05	4.1			
0.125	5.8	9.5		46
0.25	4.3	12		54
0.5	4.8	22		63
0.7	6.5	30	11	70
1.0 (control)	37	34	58	74

The effect of higher concentrations of $FeCl_3$ was studied in another experiment. A modified sea water medium (0.015 per cent KNO_3) was used with iron concentrations ranging from 0.5 to 6 µg $FeCl_3$ per ml. A duplicate set of media containing 0.01 per cent EDTA was also prepared. All the media gave similar 7 day yields. After 14 days of incubation the highest yields were obtained in media containing EDTA and 1.5 and 2 µg $FeCl_3$ per ml as shown by the following data:

$FeCl_3$ µg/ml	Cells per ml (thousands)	
	0% EDTA	0.01% EDTA
0.5	29	40
1.0	30	45
1.3	37	
1.5	31	55
1.7	30	
2.0	44	57

$FeCl_3$ µg/ml	Cells per ml (thousands) 0% EDTA	0.01% EDTA
2.5	24	
4.0		37
6.0		46

These experiments showed that sea water medium was improved by increasing the iron content and by adding 0.01 per cent EDTA.

(6) The Effect of Adding Culture Preparations

G catanella culture filtrates or autoclaved whole G catanella cultures were added to sea water media. Duplicate cultures grown in sea water media for 2 weeks were filtered through coarse filter paper and added to sea water media immediately before autoclaving. The early growth of G catanella was improved:

Filtrate	Cells per ml (thousands) 5 days	8 days	14 days
None	6.6	13	82
10% filtrate	8.7, 12	24, 27	61, 75

Growth was not further improved by increasing the concentration of the filtrate in the medium to 20 per cent. Increasing the filtrate concentration above 20 per cent often reduced the cell counts. The addition of G catanella culture filtrates which were cloudy with bacterial growth after storage at 5 C, or of autoclaved whole G catanella culture to sea water medium or sea water medium containing 0.015 per cent KNO_3 produced little or no change in the cell count. Increasing the concentration of the filtrate or autoclaved culture in the medium did not improve growth.

Suspensions of a killed mixed bacterial culture isolated from the G catanella culture and added to sea water medium gave improved growth of G catanella. The 7-day yields in control cultures and in cultures supplemented with 10 or 20 per cent of the suspension were 5,300, 11,000 and 12,000 cells per ml respectively. To determine whether one type of contanimant was responsible for this growth improvement, sea water suspensions of smooth colony types, rough colony types, and a mixture of rough and smooth types were added separately in 10 or 20 per cent concentrations to sea water medium containing 0.015 per cent KNO_3 before autoclaving. There was no difference in cell counts after 7 days; however, improvement in the presence of 20 per cent smooth type and both 10 and 20 per cent mixed culture

suspension was noted after 14 days of incubation:

Contaminant added to the medium before autoclaving	Cells per ml (thousands) 7 days	14 days
None	11	30
10% rough	12	32
20% rough	9.5	36
10% smooth	13	33
20% smooth	13	45
10% mixed	8	38
20% mixed	7.4	49

To determine whether traces of protein from the bacterial medium influenced the cell counts, 0.005 and 0.05 per cent of Bacto-peptone and pink salmon viscera meal (both of which were present in the agar medium used to cultivate the bacteria) were added separately to the modified sea water medium. Neither peptone nor fish meal altered the cell counts.

(7) The Effect of Acids

Cultures of G catanella in sea water medium remained at pH 8 during growth. Since solutions of shellfish poison are unstable at an alkaline pH, attempts were made to grow G catanella in acidified media to reduce possible poison destruction during the long growth period. Acetic acid was added to duplicate 20 ml volumes of medium in 125 ml Erlenmeyer flasks to adjust the medium to pH 7, 6 and 5. A rise in pH occurred when the acidified media were autoclaved. The amount of added acetic acid, the pH and the 12 day cell yields were:

0.1 N acetic acid added to 20 ml of medium (ml)	pH of medium Before autoclaving	After autoclaving	Cells per ml (thousands) 12 days
0	7.9	8	28
0.04	7	8	34
0.25	6	8	45
0.29	5	5.3	no growth

Growth stimulation occurred in the medium adjusted to pH 6. Cell and poison yields in media adjusted to pH 6 with acetic, hydrochloric, or sulfuric acid were compared to determine whether the improved growth was due to a nutritional response to acetate or to the presence of acid during autoclaving. The pH of these media after autoclaving was 8. The following cell and poison yields were obtained in 4 successive 7-day passages in each medium:

Acid added	Cells per ml (thousands) passage 1	2	3	4	MU per ml passage 1	2	3	4
None	33	19	22	31	0.95	0.40	0.19	0.15
Acetic	34	36	47	72	0.63	0.61	0.21	0.29
HCl	60	37	35	29	0.75	0.65	0.21	0.15
H_2SO_4	48	37	33	28	0.49	0.37	0.20	0.11

No medium consistently gave superior cell and poison yields. The outgrowth of contaminants in acetic acid treated media may have interfered with the stimulatory effects of acetic acid on G catanella.

(8) The Effect of Organic Nutrients

The effect of organic nutrients on the growth of G catanella in sea water medium was obscured by the outgrowth of contaminants present in the stock culture. Addition of polymyxin B reduced contaminant growth and improved the yields of G catanella in the control and supplemented media. The nutrients were tested at 0.01 per cent concentration in sea water medium and in sea water medium containing 20 µg of polymyxin B per ml. The results of the experiment are shown below. Numbers refer to yields of G catanella in thousands of cells per ml and the + or - signs refer to the extent of visible contaminant growth in cultures:

Supplement	Polymyxin, µg per ml 0	20
None	44 -	81 -
Autolyzed yeast	53 + +	73 +
Glucose	39 + +	52 +
Casein hydrolysate	37 + + +	30 + +
Sodium acetate	42 +	51 +
Sodium citrate	50 +	48 +

Outgrowth of contaminants was observed in all except the control cultures. Greater stimulation of G catanella occurred with polymyxin alone than with any polymyxin plus nutrient combination. It was probable that the outgrowth of contaminants in the supplemented cultures interfered with the potential stimulative effects. Raising the polymyxin level to 100 µg per ml did not prevent the outgrowth of contaminants in similarly supplemented media.

(9) The Effect of Metals, Rubber Products, Plastics or Other Materials

The construction of equipment for the production of G catanella poison would be facilitated by the use of metal or plastics in place of glass for culture vessels and culture transfer systems. Materials which might be used in the fabrication of such equipment were tested for effects on the growth of G catanella by adding 0.25 x 0.75 inch specimens to duplicate 5 ml volumes of sea water medium. The amount of growth was estimated visually after 13 days and is reported in visual growth units in Table 1.

The cultures to which galvanized sheet steel was added produced viable cells. Sheet steel specimens which were coated with either Silicone Fluid DC 200 or DC 50 (Dow Corning Company) or with liquid stainless steel (Slip On Company), likewise prevented growth, although the latter coating greatly reduced the corrosion of sheet steel by sea water. Plastic fiber glass (fiber glass reinforced with polyester resin) Tygon tubing and rubber products also inhibited growth completely. Stainless steel and polyethylene gave slight inhibition of growth, but cultures containing aluminum or Saran showed growth equal to that of the control cultures.

The metal binding agent EDTA was tested as a means of reducing metal ion toxicities, which were believed to be responsible for some of the growth inhibition observed in the above experiment. Growth in the presence of stainless steel or polyethylene was equal to the control cultures when 0.01 per cent of EDTA was added. The addition of 0.01 and 0.05 per cent of EDTA did not reduce the inhibition by galvanized sheet steel. The effect of EDTA on the inhibition by Tygon and rubber products was not determined.

The results of these experiments showed that aluminum might be used for culture vessel construction and that stainless steel or polyethylene would be satisfactory when EDTA was included in the medium. Galvanized sheet steel was unsatisfactory with or without EDTA.

A variety of materials were tested singly in sea water medium without soil extract for a protective effect on G catanella poison. The following materials were used: 0.5 per cent of either IR-4B (ion exchange resin), soluble starch, dextrin, or gelatin; 0.01 per cent of talc or coarse charcoal and 0.01, 0.05 or 0.5 per cent of celite, finely divided charcoal or agar. There was no growth of G catanella in the presence of IR-4B, starch, dextrin or gelatin. Contaminant growth was stimulated in the media containing starch, dextrin or gelatin. There was no improvement in growth or poison production of G catanella in media containing any of the other substances.

(10) The Effect of Gases

The growth of G catanella was determined in sea water medium under various gas environments. The necessity for gas exchange was shown by the

TABLE 1. THE EFFECT OF THE ADDITION OF METALS, PLASTICS, OR RUBBER PRODUCTS ON THE GROWTH OF GONYAULAX CATANELLA IN SEA WATER MEDIUM

Material added to culture	Growth (visual)		
	First passage	Second passage	
	No EDTA	No EDTA	0.01% EDTA
None	4	3	3-4
Sheet steel, galvanized	0	0[a]	0[a]
Aluminum	4	4	4
Stainless steel, No. 347	3	3-4	4
Polyethylene, sausage tubing	3	3-4	4
Saran, sheet plastic	3-4		
Plastic fiber glass	0		
Tygon tubing	0		
Gasket rubber, Garlock 353	0		
Buna N rubber	0		
Sheet neoprene, Garlock 8990	0		
Gum rubber tubing	0		

[a] These cultures were inoculated with first passage control culture, all other second passage media were inoculated with the corresponding first passage culture

absence of growth in tubes which were closed with rubber stoppers after inoculation. Growth was not initiated when the air above similar cultures was replaced with: carbon dioxide, oxygen, a mixture containing 5 per cent carbon dioxide and 95 per cent nitrogen, or a mixture containing 1 per cent carbon dioxide, 16 per cent oxygen and 83 per cent nitrogen. Likewise, only traces of growth were found in cultures incubated in closed Brewer anaerobe jars containing either air or air enriched with carbon dioxide to a concentration of 1.2 per cent. It was evident that the effects of gases could not be studied in culture systems without provisions for the replenishment and removal of gases. Growth was obtained when a slow stream of air or carbon dioxide was continuously passed over the surface of cultures; after three weeks of incubation the average cell yields of duplicate 5 ml cultures were 13,000 and 2,300 cells per ml respectively. No growth occurred in similar cultures treated with oxygen. Control cultures closed with cotton plugs gave 15,000 cells per ml. A similar experiment with 1.2 liter cultures in low form culture flasks showed no growth with 5 per cent carbon dioxide in nitrogen, and identical yields of 7,000 and 23,000 cells per ml after 17 and 24 days of incubation with moving or static air.

The results of these experiments showed that air was superior to the other gases studied in supporting the growth of C catanella and that free diffusion or flow of air was essential for good growth. The change from pH 8 to pH 6.3 and 5.3 of cultures exposed to the carbon dioxide mixtures or to carbon dioxide may account for the inhibition of growth in these cultures. The correction of this condition would permit investigations of carbon dioxide enriched cultures as superior media for the photosynthetic activities of C catanella.

(11) Growth in Sea Water Medium Containing 0.015 per cent KNO_3

Growth of C catanella was determined at frequent intervals during a 21 day incubation period in duplicate 500 ml volumes of sea water medium containing 0.015 per cent KNO_3 in Fernbach flasks, using a 10 per cent sea water inoculum. Direct cell counts on 3 or 4 samples, and cellular carbohydrate determinations by the anthrone method were made after each incubation period. The cell yields (thousands per ml) and the carbohydrate yields expressed as the optical density (OD) per ml for the 2 cultures were:

Days Incubated	Culture A Cells	Culture A OD	Culture B Cells	Culture B OD
0	6		6	
2	8.5	0.032	6	0.028
5	22	.092	18	.090
6	22	.097	16	.098
7	27	.132	19	.127
9	36	.144	25	.127
10	46	.160	28	.118
13	64	.169	44	.122
15	71		53	
17	59	.211	47	.174
21	78	.200	47	.156

The cell counts and optical density values were proportional up to 9 days of incubation. After 9 days of incubation the two methods diverged with less anthrone color per cell than during early growth. Based on cell counts, growth in sea water medium containing 0.0015 per cent KNO_3 showed a lag period of approximately 2 days followed by a period of rapid cell multiplication to maximum yields after 15 days of incubation.

b. ARTIFICIAL SEA WATER MEDIA

(1) Lyman and Fleming Medium

Growth of G catanella was obtained in Lyman and Fleming[1] artificial sea water supplemented with the same concentrations of inorganic nutrients and soil extract contained in sea water medium.

Lyman and Fleming artificial sea water contains the following percentages of inorganic salts dissolved in distilled water:

1. Jour. Maritime Res. 3, 134 (1940).

	%		%
NaCl	2.34	$NaHCO_3$	0.019
$MgCl_2 \cdot 6H_2O$	1.06	KBr	0.0096
Na_2SO_4	0.39	H_3BO_3	0.0026
$CaCl_2 \cdot 2H_2O$	0.15	$SrCl_2 \cdot 6H_2O$	0.004
KCl	0.066	NaF	0.0003

The cell yields in Lyman and Fleming medium with sea water inoculum were 11,000 and 19,000 cells per ml in two experiments compared to yields of 17,000 and 36,000 cells per ml in corresponding sea water medium cultures. The cell yields in 5 successive passages in Lyman and Fleming medium without soil extract were: 17,000; 14,000; 12,000; 0, 0, and 6,500 in triplicate fourth passage cultures; and 0 in triplicate fifth passage cultures. In a series of transfers through increasing volumes of medium, 25 ml of Lyman and Fleming medium without soil extract yielded 36,000 cells per ml after inoculation with 10 ml of sea water culture and incubation for 10 days. This entire culture was used to inoculate 175 ml of the same medium, which gave 9,600 cells per ml after 16 days of incubation. This culture was inoculated into 1 liter of Lyman and Fleming medium. The cell yields after 9, 19 and 30 days of incubation were 4,800, 6,200 and 11,700 cells per ml respectively.

In other experiments, the poison yields from first passage cultures in Lyman and Fleming media were equal to the yields in control sea water media. In general, Lyman and Fleming medium gave satisfactory growth with inocula grown in sea water media, but the depressed growth on successive subculture indicated that it could not be used exclusively in place of sea water media.

(2) Sea Salt Media

The difficulties in procuring and storing sufficient volumes of sea water for the production of G catanella poison led to further attempts to develop satisfactory artificial sea water media. An artificial sea water medium consisting of a solution of commercial sea salt in tap water supplemented with the same amounts of inorganic nutrients and soil extract as used in sea water medium gave good growth with a 15 per cent sea water inoculum. Experiments were conducted to determine the optimum concentration of sea salt in this medium prepared with and without soil extract. The cell yields in several experiments with duplicate 5 ml cultures receiving sea water inocula were:

Percentage sea salt	Cells per ml (thousands) Without soil extract	With soil extract
4	4.3, 17	
3.6	30, 20	28, 26
3.2	26	45, 34, 49
3.0	8.7	
2.4	19	40
2.0	4.7	
1.0	5.0	
Sea water medium control	24, 25	38, 36, 72

The medium containing 3.2 per cent of sea salt with soil extract (sea salt medium) was used in subsequent investigations.

(3) The Effect of EDTA in Sea Salt Media

Good growth occurred in sea salt medium with a 16 per cent sea water inoculum, but poor growth resulted upon subculture in this medium. The addition of EDTA derivatives gave improved growth in first and second passage cultures in sea salt medium. The commercial products Di Sodium Versenate (Beresworth Chemical Company) and Sequestrene Na_2 (Alrose Chemical Company) were equally effective for this purpose. In this and the following experiments with sea salt media, growth is expressed in visual units as defined above. A sea salt medium containing 0.01 per cent of EDTA gave better growth than media containing higher or lower concentrations of EDTA:

Per cent EDTA	Growth (visual) First passage	Second passage
0	2-3	0
0.001	4	1-2
0.01	4	3
0.025	3	1-2
0.05	3	0

The necessity for determining growth in subcultures of media to be evaluated was shown in this experiment. Inadequacies in the control and 0.05 per cent EDTA media were not apparent in the first passage cultures because of the carryover of nutrients with the 15 per cent sea water inoculum.

(4) The Effect of Different Marine Soil Extracts

Soil samples from the Pacific Ocean (Iverness tideland mud[1]), from Alaskan poison clam beds[2], and from the Atlantic Ocean at Norfolk, Virginia[2], gave extracts with similar activity. The extracts were assayed at a 2 per cent by volume concentration in sea salt medium and in this medium with 0.01 per cent of added EDTA. The first passage media received a 16 per cent sea water inoculum. The second passage media were inoculated with the corresponding first passage cultures (14 day) and incubated 11 days. Control media without soil extract were included in both passages to give a measure of soil extract stimulation. The following results were obtained:

Soil extract source	Growth (visual)			
	First passage		Second passage	
	- EDTA	+ EDTA	- EDTA	+ EDTA
Control	3-4	4	1	1-2
Pacific mud	4	4	2	3-4
Alaskan clam bed	4	4-5	1-3	3
Norfolk, Va., harbor	4	4	1-2	3-4
Norfolk, Va., tide wash	4-5	4-5	2-3	3-4

The four soil extracts gave similar stimulation in the second passage cultures, with the best growth occurring in the presence of both soil extract and EDTA.

The activity of the inorganic cations in soil extract was studied by ion exchange resin techniques. A 50 ml sample of marine soil extract was passed through a 0.75 in. by 7 in. column of Dowex-50 followed by 25 ml of redistilled water. Analyses on the combined effluent (decationized soil extract) showed that Dowex-50 treatment reduced the total solids of the soil extract sample from 0.96 to 0.13 per cent, and the ash from 0.1

1. Collected by R. E. Mills, Hooper Foundation, San Francisco, Cal.
2. Obtained by R. R. Rowl, HM1, USN.

per cent to a negligible level. Fifty ml of 2 N HCl followed by 25 ml of redistilled water were passed through the column to remove the soil extraction cations. The stimulatory activities of equivalent volumes of untreated soil extract and neutralized samples of the Dowex-50 fractions were compared in sea salt medium with sea water inoculum. Decationized soil extract gave first passage growth stimulation equal to untreated soil extract. The cation fraction did not stimulate growth. Several metallic cations were tested for possible stimulation of the decationized soil extract. Calcium (50 µg per ml) was inactive, and magnesium (50 µg per ml) and manganese (10 µg per ml) reduced the growth. The mixture of inorganic trace nutrients devised by Hoagland was also inactive and supported the conclusion that inorganic cations were not the source of soil extract stimulation.

(5) The Stimulatory Effect of Sea Water in Sea Salt Media

The reduction in growth of G catarella with successive passage in sea salt media was overcome by the addition of small quantities of sea water. Similar growth stimulation was obtained with the filtrate of concentrated sea water which suggested that the required sea water nutrients remained in the concentrated brine in the commercial sea salt production process. The laboratory concentration of sea water was carried out in the following manner: 500 ml of sea water was concentrated to 10 ml on a steam bath and transferred to a filter. The solids on the filter (laboratory sea salt) were washed with distilled water to bring the filtrate (brine) volume to 25 ml. The effect was studied of including 5 per cent of brine or 15 per cent of sea water in sea salt media prepared with commercial sea salt or with laboratory sea salt. Samples of 11-day first passage growth in each medium were used to inoculate fresh tubes of the same medium. The following growth resulted in the second passage cultures:

Supplement	Growth (visual)			
	Commercial sea salt		Laboratory sea salt	
	7 days	14 days	7 days	14 days
None	1-2	2	1-2	2-3
Brine	2	3-4	2-3	3-4
Sea water	3-4	3-4	1-2	4

It was evident that the brine gave growth stimulation similar to sea water in both sea salt media.

To determine whether this stimulation could be replaced by soil extract, sea salt media containing different concentrations of soil extract or of sea water were prepared. The inoculum consisted of cells grown in sea water medium which were collected and washed on coarse paper by

gravity filtration and resuspended in sea salt medium. This treatment greatly reduced the number of motile cells in the inoculum and prolonged the lag period of growth. The following growth was obtained after 14 and 21 days:

Medium		Growth (visual)	
% soil extract	% sea water	14 days	21 days
0	0	0	0
2	0	0	0
4	0	0	0
6	0	0	0
10	0	0-1	0-1
2	5	1	1-3
2	10	1-2	3
2	15	2-3	4
sea water medium	97.6	2	3-4

The growth stimulation shown by sea water was not replaced by soil extract. The washed inoculum technique was effective for obtaining valid data with first passage cultures.

An experiment testing inorganic trace elements as the source of the sea water stimulation was in progress at the termination of this project. Several metallic cations were added singly to sea salt medium. The first passage cultures received sea water inoculum and distinct growth effects were not obtained. A decreased lag period was indicated in the media supplemented with ionic Ca, Mg, Mn, Zn or Co. No effect was observed with ionic Al, Ni, Cu, Li or Mo. A mixture of inorganic trace nutrients containing all of these ions did not promote growth in sea salt medium inoculated with washed cells. The same inoculum grew in medium supplemented with 5 per cent of sea water. Therefore, it seemed advisable to add 5 to 15 per cent of sea water to sea salt medium. The use of such media in place of sea water media would be advantageous for poison production by reducing the collection, transportation and storage of sea water by at least 85 per cent.

5. PURIFICATION OF THE GONYAULAX CATANELLA STOCK CULTURE

a. INTRODUCTION

Undesirable outgrowth of the bacterial contaminants present in the stock culture of G catanella occurred in cultures held for more than 2 weeks without transfer and in younger cultures grown in experimental media supplemented with organic nutrients. When the contaminant population became too large, the G catanella cells did not survive. Several methods of eliminating the contaminants in suspensions of actively motile G catanella cells were tried: inhibition of contaminants by treatment with antibiotics, separation by washing procedures, and elimination of contaminants by treatment with ultraviolet light. The presence of bacterial contaminants in the treated G catanella culture was determined by streaking on sea water agar (sea water peptone agar fortified with fish meal extract). The plates were incubated at room temperature and examined for bacterial colonies after 24, 48, and 72 hours. G catanella did not grow on any solid media tried. In no case, using one or a combination of purification techniques, were the bacterial contaminants entirely eliminated; bacterial cells capable of eventually outgrowing the Gonyaulax always remained.

b. THE EFFECT OF ANTIBIOTICS

(1) Treatment of Isolated Contaminants with Antibiotics

The bacterial contaminants present in the G catanella culture were divisible on the basis of colony appearance on sea water agar into three general groups: (1) those forming "rough" nonpigmented colonies, (2) those forming "smooth" nonpigmented colonies and (3) those forming "smooth" pigmented colonies. Pink colonies of type (3) appeared on agar after 2 to 3 weeks of incubation, while types (1) and (2) produced visable colonies after 24 to 48 hours of incubation. Many colonies, belonging to all three types, darkened on aging. The lag phase of growth for all types of contaminants was often longer when subcultured from a liquid culture containing high levels of antibiotics. Young cultures of the above contaminants were short Gram-negative rods; however, older cultures often stained irregularly with the Gram stain. Pleomorphic forms were often seen, especially in the presence of high concentrations of some antibiotics. Type (1) organisms were the most susceptible to the inhibiting effects of all of the antibiotics used, type (3) were next in susceptibility and type (2) were the least susceptible. In many cases the presence of an antibiotic in a medium kept the bacterial population to a minimum because of inhibition but not to death of these cells; however, subculturing into fresh media without antibiotics allowed the bacteria to multiply again.

To determine the most efficient concentrations of antibiotics which would inhibit the bacterial contaminants, pure cultures of types (1) and (2), isolated from the mixed culture, were seeded on separate petri plates containing sea water agar by spreading 0.5 ml of a sea water suspension of

each bacterial culture with a sterile glass rod. Sterile 1.8 cm filter paper discs were placed on the seeded plates and 0.1 ml of increasing concentrations of the antibiotics or brilliant green were placed aseptically on the filter papers. These plates were incubated overnight at room temperature. Inhibition of the bacterial growth was demonstrated by zones of inhibition around the filter paper discs. Both contaminants were inhibited by the following: 100 µg per ml of aureomycin or penicillin G; 10 and 100 µg per ml of streptomycin or polymyxin B; 4, 40, and 400 units per ml of streptothricin; and 1000 and 10,000 µg per ml of brilliant green. Actidione, 0.01 to 100 µg per ml, did not inhibit either contaminant.

(2) Treatment of the G catanella Culture with Antibiotics

Antibiotics or brilliant green added separately to G catanella cultures did not eliminate the bacterial contaminants. G catanella grew in test tubes containing 5 ml volumes of sea water medium when 0.1 to 100 µg per ml of the following antibiotics were added aseptically: actidione, aureomycin, circulin sulfate, polymyxin B, crude polymyxin, streptomycin, terramycin, antibiotic-S (Abbott), A686-3 (Bristol), chloromycetin, neomycin or viomycin. G catanella also grew when 4 to 400 units per ml of streptothricin or 1 to 1000 units per ml of penicillin G were added aseptically to sea water medium. Brilliant green (10 to 10,000 µg per ml) and penicillin G plus procaine completely inhibited the growth of G catanella in sea water medium. The "rough" colony type was inhibited by all antibiotics used except actidione and penicillin G. With streptomycin, polymyxin B, circulin sulfate, and A 686-3 all bacterial contaminants were reduced to what was considered just one type (type 2).

The addition of antibiotics to sea water medium without soil extract or to Lyman and Fleming medium without soil extract affected the cell yields of G catanella as shown in Table 2. Streptothricin, streptomycin, penicillin and polymyxin improved growth of G catanella. Aureomycin (20 to 100 µg per ml) or circulin sulfate (200 µg per ml) inhibited G catanella.

(3) The Effect of Antibiotics on Poison Production

When 20 ml volumes of antibiotic-treated G catanella cultures were inoculated into 50 ml of sea water medium, poison was present in all cultures after 9 days of incubation. The highest cell counts and toxicity were obtained with the culture treated with circulin sulfate. One culture yielded 51,000 cells per ml and 0.52 to 0.77 MU per ml culture and the duplicate culture yielded 46,000 cells per ml and 0.97 to 1.4 MU per ml culture.

(4) Serial Passage of G catanella in Media Containing Antibiotics

Serial passage of G catanella in the presence of one antibiotic did

TABLE 2. THE EFFECT OF ANTIBIOTICS ON THE GROWTH OF GONYAULAX CATANELLA IN MEDIA WITHOUT SOIL EXTRACT

Antibiotic concentration	Cells per ml (thousands) Exp. 1[a]	Exp. 2[a]	Exp. 3[b]
None	25	14	6.5
Streptothricin 8 units/ml		23	
" 80 "		23	
Streptomycin 5 µg/ml		21	
Penicillin G 1 unit/ml	40		
" 10 "	26		
" 100 "	21		2
Polymyxin B 10 µg/ml			14
" 20 "		10	
" 100 "		8.5	31
" 200 "		9	
Circulin 5 µg/ml sulfate		13	
" 50 "		10	
" 100 "		12	
" 200 "		4	
Aureomycin 20 µg/ml		7	
" 100 "		1	

a. Exp. 1 and 2 - Cells grown in sea water medium without soil extract.

b. Exp. 3 - Cells grown in Lyman and Fleming medium.

not eliminate the contaminants. The G catanella cells at the surface of cultures in test tubes were transferred at weekly intervals in sea water medium without soil extract but containing one of the following antibiotics: 8 or 80 units of streptothricin per ml; 5 μg streptomycin per ml; 1, 10 or 100 units of penicillin G per ml; 10, 20, 100 or 200 μg polymyxin B per ml; 5, 50, 100 or 200 μg circulin sulfate per ml; and 20 or 100 μg aureomycin per ml. G catanella did not survive after the second transfer in media containing aureomycin or streptomycin. Reduced growth of G catanella occurred in the presence of the other antibiotics after 4 transfers; however, no culture was free of contaminants.

The growth of G catanella in sea water medium without soil extract but containing mixtures of 3 antibiotics (60 or 100 μg of polymyxin B, 8 units of streptothricin, and 100 μg of circulin sulfate per ml) decreased with each transfer and did not survive the third serial passage. The omission of soil extract in the media used for serial passage may have been responsible for the reduction in growth.

c. ATTEMPTS TO REMOVE BACTERIAL CONTAMINANTS BY WASHING TECHNIQUES

An attempt was made to separate G catanella from the contaminants by rapid serial transfer in Lyman and Fleming medium. The G catanella culture was first subcultured in sea water media containing antibiotics to reduce the contaminant population as follows: 4 transfers in the presence of 8 units of streptothricin or 100 μg of circulin sulfate per ml, followed by 1 transfer in the presence of a mixture of these antibiotics. Active cells from the upper surface of the culture were transferred to Lyman and Fleming medium and 12 similar transfers were made at 45 to 60 minute intervals in 5 ml volumes of Lyman and Fleming medium. After 7 days of incubation G catanella cells were present in only the first and second subcultures. Contaminants were detected in subcultures 1 through 10 by inoculation into enriched media.

Rapid serial transfer of a protozoan cell through sterile droplets of media was used by Glaser and Cori[1] to obtain a bacterial free culture of the protozoa. In repeated attempts at single cell manipulation of G catanella in a micropipet using a widefield microscope, the Gonyaulax cells were not motile after the second transfer even when a cold stage was used or all transfers were made in the 17 C laboratory. To determine whether a very small inoculum could initiate growth, 1 or 5 cells were transferred to droplets of sea water medium on a series of slides and these slides were incubated in an illuminated moist chamber. All cells were nonmotile after 2 days and there was no increase in cell number after 2 weeks of incubation. To reduce the effects of evaporation of media during incubation, several cells were transferred by micropipet directly into 0.5 ml of sea water medium in a test tube. There were no G catanella cells in these tubes after 2 months of incubation.

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1. R. W. Glaser and M. A. Cori, Jour. Exp. Med. 51, 787 (1930).

A washing technique based on the difference in cell size between G catanella and the bacterial contaminants utilized a filter which would retain the larger G catanella cells on a layer of glass beads and allow the smaller bacteria to be washed into the filtrate. In general the filters consisted of 18-mm tapering pyrex tubing, 3 to 26 cm long, containing a lower layer, 0.5 or 5.0 cm in depth, of glass beads 200 μ in diameter and an upper layer, 0.5 to 1.5 cm in depth, of glass beads 100 μ in diameter. All washing was performed at 17 C. The filter was fitted to a sterile suction flask, a sample of G catanella culture was placed on the filter, and sterile sea water allowed to flow through the filter. The washing was discontinued at hourly intervals and the culture illuminated for 30 minutes to stimulate the Gonyaulax cells to swim to the surface of the liquid above the filter. Samples of the culture above the filter were removed and inoculated into sea water medium with or without antibiotics. Bacteria-free G catanella cultures were not obtained even when the following improvements and modifications were made: the Gonyaulax cells to be washed were passed through two transfers at weekly intervals in sea water medium containing circulin sulfate to reduce the initial bacterial population; the Gonyaulax culture was rewashed; the washing of the cells was hastened by applying gentle suction; the washed culture samples were inoculated into sea water medium containing antibiotics known to inhibit some of the bacterial contaminants present (polymyxin B, circulin sulfate, streptomycin or streptothricin). Finally a G catanella culture, grown in the presence of circulin sulfate, was rewashed several times and each sampling of the washed cells transferred to sea water medium and sea water medium plus polymyxin B, circulin sulfate, streptomycin or streptothricin. The bacterial contaminants were not completely eliminated by this procedure.

The number of bacteria was probably greater than the number of G catanella in cultures one week old, even when the usual growth of G catanella was obtained. A washing procedure designed to dilute the bacteria and not the G catanella cells was based on the fact that when sterile sea water was added to the impure culture, G catanella cells would swim to the surface of the liquid medium, allowing the bacteria in the lower layer to be continuously diluted and removed. When a G catanella culture was washed with three, 500 ml volumes of sterile sea water in a 1000 ml dispensing cylinder both G catanella and bacteria survived. A 250 ml leveling bulb was then substituted for the cylinder. Both G catanella and bacteria were recovered after washing with 1 liter of sea water but continued washing yielded only bacteria.

d. TREATMENT WITH ULTRAVIOLET LIGHT

G catanella cells were found to be more sensitive to ultraviolet light than the bacterial contaminants. A thin layer of G catanella culture in the well of a sterile culture slide was exposed to ultraviolet light at a distance of 4 inches for 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6 and 7 minutes. G catanella cells survived and grew on subculture in

sea water medium after exposure for 1 to 3.5 minutes and in one trial for 4 minutes. Bacterial contaminants survived all periods of ultraviolet treatment.

6. THE PRODUCTION OF GONYAULAX CATANELLA POISON

a. STATIONARY CULTURES

Poison production was studied in increasing volumes of sea water medium to determine the conditions for maximum poison yields with the available 17 C facilities. A decrease in poison yield resulted as the depth of the culture increased as shown by the maximum yields obtained with various volumes of medium in different culture vessels:

Volume, ml	Depth, cm	Cells per ml (thousands)	MU per ml
20	1.5	33	0.95
70	2.5	49	1.2
200	2.5	19	0.57
600	7.0	18	0.22
1,400	4	17	0.48
1,400	11.6	10	0.26
2,000	5.0	21	0.24
11,000	18	5.7	0.15

The best yields were obtained in shallow cultures. Yields in 18 cm deep cultures were not significantly improved by keeping the culture mixed by a magnetic stirrer or stream of air bubbles. The better yields in shallow cultures probably resulted from superior illumination and aeration of the medium.

b. ROTATING CULTURES

A rotating fermenter was tested as a means of improving the aeration

and illumination of cultures in horizontal 20 liter pyrex bottles. Growth and poison yields were determined in 3.5 liter cultures illuminated with five 15-watt fluorescent tubes placed 1 inch from the outer surface of the bottle. A series of experiments gave the following results:

Rotation speed	Cells per ml (thousands) days						MU per ml days					
rpm	3	5	7	9	10	11	3	5	7	9	10	11
3		13	21		36			.09	.19		.24	
			10	26	34	35			.11	.13	.13	.24
	15	22	36	47	62	50	.17	.09	.12	.18	.09	.06
				33						.30		
8		5.3	24						.56			

The poison yields of 0.3 and 0.56 MU per ml represented increases over previous yields from cultures of this volume. The advantage of the rotating fermentor was probably due to improved illumination and aeration of the film of culture carried on the inner surface of the rotating bottle.

The effect of illuminating with a single 36 inch, 30 watt fluorescent tube placed inside the rotating (3 rpm) bottle was studied as a means of utilizing the fluorescent light more efficiently and of providing illumination in opaque rotating tanks proposed for production use. A stationary control culture illuminated the same way was included in each experiment. The composition of the medium and the volume were the same as in the preceding experiments with outside illumination. In the first experiment unjacketed fluorescent tubes were used with the following results:

	Cells per ml (thousands) 4 days	8 days	MU per ml 4 days	8 days
Rotating culture	9	19	.45	
Stationary culture	8	21	.55	.64

The poison extract from the 8-day rotated culture was lost, but the similarity of the other yield data indicated that rotation did not affect the yields. In a second experiment the fluorescent tube was jacketed with a glass tube and the annular space between the two tubes (5 mm) was filled with water as a filter to remove possible harmful radiation. The results of this experiment were:

	Cells per ml (thousands) 6 days	8 days	MU per ml 6 days	8 days
Rotating culture	8	17	.36	.34
Stationary culture	9	18	.43	.29

The growth and poison yields were smaller than with the unjacketed tube; therefore, no benefit was realized by filtering the light through water and glass. The experiments with inside illumination showed equally low yields in stationary and rotated cultures. It was possible that the growth of contaminants in these cultures may have limited G catanella cell and poison yields. Studies on the use of antibiotics to check contaminant growth in rotating cultures were in progress at the termination of the project. The addition of 1 µg of polymyxin B per ml of sea water medium containing 0.015 per cent KNO_3 gave poison yields after 7 days of incubation of 0.48 and 0.13 MU per ml respectively in rotated and stationary cultures. The effect of 20 µg of polymyxin per ml of sea water medium containing 0.015 per cent KNO_3 was determined under several conditions of incubation. Each bottle containing 3 liters of medium was inoculated with 500 ml of the above rotated culture which reduced the polymyxin concentration to 17 µg per ml. The following yields were obtained:

Incubation	Polymyxin µg per ml	Illumination	Cells per ml (thousands) 4 days	7 days	MU per ml 4 days	7 days
Rotated	17	outside	16	48	0.81	1.3
Stationary	17	outside	16	37	.34	.52
	0	outside	21	34	.31	.33
Stationary	17	inside	7	16	.19	.38
	0	inside	13	17	.24	.35

The results of this experiment indicated that polymyxin, rotation and outside illumination were desirable for poison production. The poison yields obtained in the rotated culture represented substantial gains over previous yields. With an average yield of 1 MU per ml per week the total poison production from twenty, 3.5 liter cultures (capacity of the 17 C laboratory) would be 70,000 MU per week. This estimate probably does not represent the ultimate poison production with the existing facilities. Additional gains in poison yield would be expected with further research on the use of antibiotics or purified cultures, and on the improvement of

the culture medium. In view of these considerations it appears that the laboratory production of C catanella poison for chemical studies is feasible.